MECHANISM OF PROTEINURIA

IV. Effect of Renin on Hemoglobin Excretion*

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(Received for publication, February 1, 1951)

The observation that renin administration induces proteinuria in the rabbit was made in 1940 by Pickering and Prinzmetal (1). The proteinuria was associated with a pronounced diuresis and a parallel increase in sodium and chloride excretion. These effects were attributed to a direct action on the renal tubules since the effects persisted after tachyphylaxis, when the pressor effect was absent.

Subsequently Brandt and Gruhn (2) reexamined the phenomenon and confirmed the original observation. They also tested the effects of renin administration upon hemoglobin excretion in the rabbit. From a small number of observations, they concluded that renin administration did not increase glomerular permeability to hemoglobin in the rabbit. From this opinion, they further inferred that the proteinuria resulted from the inhibition of tubular protein absorption.

Addis and others (3) found that the administration of renin also produced proteinuria in the rat. Since the proteinuria paralleled the pressor action, and repeated injections of renin at proper intervals resulted in a diminished proteinuria response, analogous to tachyphylaxis, it was believed that renin proteinuria in the rat is dependent on the pressor action. In addition, Addis noted that a clot formed in the urine collected after renin administration. This suggested to him that the glomerular membrane had permitted the passage of fibrinogen, as the result of increased permeability to protein.

Rather and Addis (4) found that renin injections greatly increased the athrocytosis of hemoglobin and bovine albumin in the rat kidney. In these experiments, direct observation revealed that renin did not appear to diminish tubular hemoglobin absorption in the rat, but actually increased the number of droplets visible in cells of the proximal tubules.

In view of these conflicting observations, an investigation was undertaken with complementary physiologic and morphologic methods that have been used for the analysis of other types of proteinuria (5).

* The authors gratefully acknowledge the technical assistance of Ruth Taniguchi. The portion of this work which deals with renal function was aided by a grant from the National Heart Institute of the National Institutes of Health, Public Health Service (H 132 C2); the morphologic work by a grant from the Life Insurance Medical Research Fund.

‡ Fellow of the John Simon Guggenheim Memorial Foundation.

Plan of the Work

From *a priori* considerations, four major factors would seem to govern the rate of urinary protein excretion: the glomerular filtration rate, the glomerular membrane permeability, the tubular absorption rate, and the rate at which the tubular epithelium can dispose of absorbed protein. An indication of the glomerular filtration rate can be obtained in the rat from the exogenous creatinine clearance (6). The glomerular permeability to protein and the tubular absorption rate can be estimated by the use of hemoglobin as an indicator (5), as will be described.

A direct indication of glomerular permeability and tubular absorption can be obtained by the direct histologic observation of hemoglobin, as made visible by a modified benzidine stain. With this information, some conclusions can be drawn concerning the rate and mechanism of tubular disposal of absorbed hemoglobin.

A. FUNCTIONAL ASPECTS

Methods and Materials.—In the investigation of function, 84 rats of the Slonaker strain were used, each weighing about 150 gm. Hemoglobin and creatinine clearances were determined simultaneously by the "undisturbed" method (7), with the minor modifications indicated (5). Since this experiment was performed concurrently with our previous study, and with the same plan, the control groups are the same ones used before.

All the groups received three intraperitoneal injections, at 9.30 a.m., 4.30 p.m., and 9.30 a.m. the following morning. Each injection consisted of 16 ml. 0.85 per cent solution of sodium chloride. Hemoglobin was administered intravenously 5½ hours after the third intraperitoneal injection of sodium chloride solution, and the dose of hemoglobin was varied by diluting 7 per cent hemoglobin solution[1] with 7 per cent human albumin, so that the total volume of protein injected was always 1.6 ml. The creatinine clearance was determined simultaneously by the addition of 0.10 ml. of 5 per cent creatinine solution to the hemoglobin dose. The intravenous injection was performed under light ether anesthesia, and one group of animals was killed by exsanguination from the abdominal aorta 2 minutes after the hemoglobin injection. An identical, paired group of animals was given 10 per cent dextrose solution to drink *ad libitum* for 15 minutes, while a urine collection was made, and they were then killed in the same way.

The groups studied for the effect of renin received 4 Goldblatt dog units of renin, incorporated in the hemoglobin solution for intravenous injection. The renin used[2] was a highly purified preparation, designated K_{144}, which contained 7.7 units per mg. Another preparation, designated K_{151}, was used in the last group and contained 4.3 units per mg.

For reasons presented on several occasions (5, 7), we have reported rates of excretion in terms of the predicted kidney weight in grams (GKWP) (8). If our rates are multiplied by the factor 0.73, a rough equivalent to the rate per 100 gm. body weight is obtained.

Total hemoglobin concentration was measured by the method of Evelyn and Malloy (9).

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[1] The hemoglobin solution was from the same lot used in our earlier work (5), and was prepared by Dr. R. B. Pennell of Sharp and Dohme, Inc. The human albumin was diluted with 0.85 per cent sodium chloride solution from concentrated salt-poor, human albumin donated by The American National Red Cross.

[2] Renin was provided generously for our use by Dr. Erwin Haas.

Creatinine determinations were performed by the method of Bonsnes and Taussky (10). Mean hemoglobin and creatinine concentrations were calculated by assuming an exponential rate of fall in concentration during the experimental period (7).

RESULTS

The administration of renin resulted in a moderately pronounced diuresis. The urine collections during a 15 minute period had a mean of 2.7 ml. compared with 1.7 ml. for the control groups. It was obvious that there had been a sharp, absolute increase in the excretion of hemoglobin, since the urine, which exceeded the control specimens in volume, also had a much deeper red color. There was a significant drop in the creatinine clearance, tested by the criteria of Fisher (11), as the mean control value was 1.36 ml./min./gm. predicted kidney weight (GKWP) while the animals that received renin had a mean creatinine clearance of 0.83 ml./min./GKWP. The excretion rates are recorded in Table I.

TABLE I

Simultaneous Determination of Creatinine Clearance, Hemoglobin Excretion and Serum Concentration

KWP	Urine volume	Hemoglobin				Creatinine					*FS* col. (1) × col. (2)
		Initial concentration	Final concentration	(1) Mean concentration	Excretion	Initial concentration	Final concentration	Mean concentration	Excretion	(2) Clearance	
mg.	*ml./15 min.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	*mg./min./GKWP*	*mg./ml.*	*mg./ml.*	*mg./ml.*	*mg./min./GKWP*	*ml./min./GKWP*	*mg./min./GKWP*
					Controls						
1285	1.6 ± 0.4*	2.22	1.84	2.05	0.0742	0.0731	0.0412	0.0557	0.0796	1.44 ± 0.30*	2.95
1277	1.8 ± 0.7	6.65	5.18	5.99	0.334	0.0750	0.0379	0.0561	0.0766	1.37 ± 0.11	8.21
1306	1.7 ± 0.7	10.06	7.90	9.09	0.515	0.0869	0.0393	0.0621	0.0771	1.26 ± 0.21	11.45
Mean.	1.7 ± 0.6									1.36 ± 0.22	
					Renin						
1064	1.8 ± 0.8	1.77	1.76	1.77	0.0314	0.1269	0.0595	0.0922	0.0322	0.37 ± 0.22	0.65
1049	3.5 ± 0.9	3.01	2.53	2.80	0.248	0.1247	0.0510	0.0853	0.0840	1.00 ± 0.20	2.80
1060	2.4 ± 0.4	8.40	7.43	7.98	0.495	0.1240	0.0619	0.0924	0.0635	0.69 ± 0.08	5.51
1064	3.0 ± 0.8	12.00	8.28	10.26	1.082	0.1155	0.0266	0.0554	0.0673	1.25 ± 0.31	12.82
Mean.	2.7 ± 0.8									0.83 ± 0.31	
	$P = 0.015$‡									$P = < 0.01$	

* Standard deviation of the mean (6 determinations in each group).

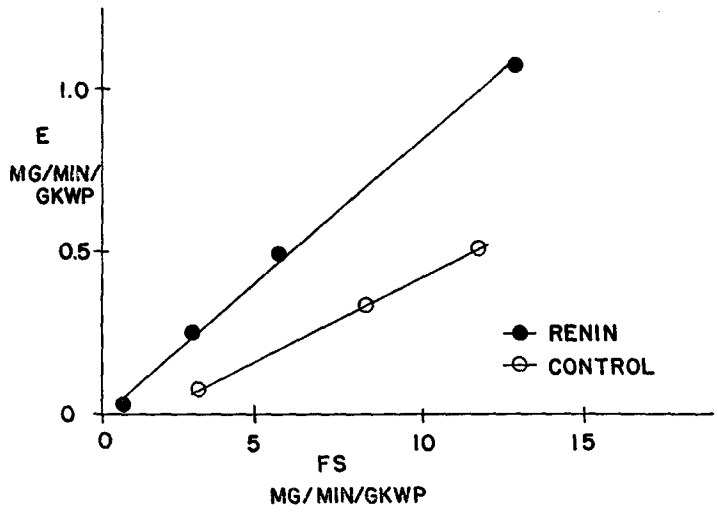
‡ *P* calculated by method of Fisher (11) to indicate significance of difference from mean value of controls.

When the hemoglobin excretion rates are plotted against the product of serum hemoglobin concentration and creatinine clearance (Text-fig. 1), it is clearly seen that the effect of renin has been to increase sharply the slope of the line, which runs through the origin and does not have a negative *y*-axis intercept. In the light of previous discussion concerning the significance of such a

plot (5), in which the negative y-axis intercept is a measure of tubular absorption and the slope is a measure of glomerular permeability, provided certain justifiable assumptions are made, it would appear that the administration of renin has increased glomerular permeability to hemoglobin while diminishing the tubular hemoglobin absorption.

B. STRUCTURAL ASPECTS

The excretion of certain proteins in the urine is accompanied by the appearance of droplets in the renal cells in a specific portion of the proximal convolution. These droplets are in part composed of the protein that is being excreted



TEXT-FIG. 1. Effect of renin administration upon the relation of hemoglobin excretion rate (mg./min./predicted kidney weight in gm.) to the product of glomerular filtration rate and serum hemoglobin concentration.

and in part of certain cytoplasmic constituents of the renal cells, for example ribonucleic acid and phospholipids. The latter materials are presumably derived chiefly from the mitochondrial rodlets which disappear as the droplets form. The structural picture thus evolved is interpreted as one morphologic aspect of protein absorption (12).

It has already been shown by functional methods that preliminary injections of bovine albumin increase glomerular permeability to the passage of hemoglobin and reduce its absorption by the tubules (5). In contrast, the injection of egg white proteins has no such effects. On morphologic examination, in the former case, the tubular lumina were found to contain collections of hemoglobin in high concentration, the result in part of increased filtration, but the tubule cells filled with bovine albumin droplets contained no droplets of hemoglobin.

In the case of egg white proteins, there was no excess of hemoglobin in the tubular lumina and the droplets of egg white proteins were deeply tinged with absorbed heme pigment. There was, therefore, a satisfactory correlation of the functional and morphologic aspects of handling of the various proteins by the nephrons (5).

In the present experiments, functional examination has shown that renin produces a sharp increase in filtration of hemoglobin and a decrease in its tubular absorption and hence resembles that of preliminary injections of bovine albumin in its general effect. The situation in the tubule cells must be quite different in the two cases, however, for after administration of bovine albumin the cells are filled with albumin droplets which do not combine with hemoglobin, but which interfere with its absorption. One would not anticipate such "physical" interference after renin administration, for no droplets of this substance are formed in the renal cells. This circumstance offers an opportunity to examine and correlate in its morphologic and functional aspects another and perhaps contrasting example of the mechanisms of proteinuria. A similar experimental procedure to that which proved useful in our previous experiments will be followed, namely to observe by histochemical means the distribution of hemoglobin in the renal cells and tubules of animals treated with renin and compare the findings with what is known to occur in normal control animals and in animals treated with preliminary protein injections, for example bovine serum albumin and egg white.

Methods.—A total of 36 rats was used in experiments that followed the general plan of the functional study. 5½ hours after the last intraperitoneal injection of sodium chloride solution, each animal received an intravenous injection of 2.0 ml. 7 per cent hemoglobin. An hour later this injection was repeated and after another hour the animals were killed. Those animals which were studied for the effect of renin received 3 units of renin incorporated in each hemoglobin injection. In addition, they received 4 units of renin in a volume of 1.00 ml. by intraperitoneal injection simultaneously with the first intravenous injection, and 1 unit of renin in a volume of 0.25 ml. simultaneously with the second intravenous injection, in order to prolong the renin effect.

The control experiments were of two sorts. In one set, the renin administration was omitted. In another set the injections of hemoglobin were replaced by injections of saline solution.

Because of the possibility that species may differ in their response to renin injection and because so much previous work has been done in the rabbit, morphologic studies were performed upon 9 female Belgian rabbits, each weighing about 1.8 kg. These were divided into four groups. One group received an intravenous injection of 11.0 ml./kg., 7 per cent hemoglobin in which was incorporated 23 units of renin/kg. After 30 minutes, these animals received another intravenous injection of 23 units renin/kg., and 1 hour after the first injection they were killed by the intravenous injection of air. A control group received the same injections, except that saline solution was substituted for the renin. Another group received three intravenous injections at 30 minute intervals, each containing 5.0 ml./kg. 7 per cent hemoglobin in which was incorporated 2.5 units renin/kg., and 2 hours after the first injection they were killed. A second control group received the same injections, except that saline solution was substituted for the renin.

When the rabbits were killed, the difference between the control animals and those which had received renin was obvious. In the controls, only a small amount of dark red urine had been voided. The bladder was small and contained a few milliliters of dark red urine. In the rabbits which had received renin, there was a profuse diuresis and the bladders were distended with light red urine. The kidneys of the latter animals were slightly darker in color than the kidneys of the controls.

The kidneys of the rats were sliced sagittally and fixed in 10 per cent formalin. The rabbit kidneys were sliced sagittally and a central segment was removed for fixation in 10 per cent formalin. Paraffin sections were stained by the benzidine method of Ralph (13).

RESULTS

As a result of its absorption by the tubule, hemoglobin, when stained by benzidine, may appear in the cells of the proximal convolutions in two forms: as fine (1 to 2 μ), somewhat irregular, bright yellow droplets, and as coarse (2 to 5 μ), deeply tinged droplets. The fine droplets of our previous experiments (5), were observed in a normal, control animal when it was given an intravenous injection of hemoglobin without renin. The coarse droplets were sharply limited to the cells which lay in immediate contact with tubular lumina that contained hemoglobin in a very high concentration and were found when increased glomerular permeability and decreased absorption had caused the patchy accumulation of concentrated hemoglobin in the tubules.

In our present experiments the cells of the cortical proximal convolutions of the control animals, which had received hemoglobin without renin, were filled with fine droplets and there were no collections of the pigment within tubular lumina (Fig. 1).

In some of the animals which had received hemoglobin and renin many of the glomerular spaces and the tubular lumina contained heavy concentrations of pigment; around the tubular collections were seen coarse droplets of varying size. In other animals the intratubular collections of hemoglobin were infrequent and there was a corresponding decrease in the number of coarse droplets.

In all the animals which had received renin, the cortical proximal convolutions that did not contain hemoglobin within their lumina were filled throughout with fine droplets, as many as in the similar tubules of the control animals (Fig. 2).

In summary, there were as many, and at times more, droplets of hemoglobin within the cells of the proximal convolutions of the animals that received renin as in those of the controls.

DISCUSSION

Upon first consideration, the results of our functional and morphologic studies of hemoglobin excretion after renin administration in the rat seemed to be paradoxical with respect to tubular absorption. Both methods of examination indicate that glomerular permeability is sharply increased. However, while the physiologic measurements indicate that tubular absorption has been greatly

diminished, if not completely inhibited, morphologic examination shows that, unlike tubule cells which have first been filled with bovine albumin to inhibit hemoglobin absorption (5), the proximal tubule cells of the present experiments contain large numbers of hemoglobin droplets, certainly no less than the control specimens without renin administration.

It is obvious that the absorption-disposal process concerned in the handling of any absorbed material must be a complex of several factors: passage of the absorbed material through the internal, luminal membrane; concentration within the cells; transport through the cell body; passage out through the external cellular membrane; and passage through the capillary or lymphatic endothelium. Previously, in considering transport through the cell body, it has been tacitly assumed, following Rather (14), that the hemoglobin observed in droplet form constitutes the major form in which hemoglobin passes through the tubule cell, and therefore that the number of visible droplets is an index of the amount of absorption.

In the absence of any knowledge concerning the details of absorption it is helpful to consider what is known concerning the mechanism by which certain substances are passed through the tubule wall in the opposite direction. The morphologic and functional aspects of the "secretion"[3] of neutral red have been analyzed by the "extravital" method in the perfused frog's kidney (15).

The intracellular structural alterations that occur during the secretion of neutral red are identical with the changes that occur when a protein is absorbed. The filamentous mitochondria dissolve as the formation of intracellular droplets of neutral red takes place. These droplets can be shown to contain a combination of mitochondrial substance and neutral red, since they stain with Janus green. One cytoplasmic constituent of the droplets is ribonucleic acid for they are strongly Gram- and pyronine-positive. Similar cytologic changes occur in the renal cells when proteins are absorbed (12) and this structural identity in the two processes, secretion and absorption, suggests a similarity in their functional mechanisms.

In the perfused frog's kidney, it has been shown by functional examination that the transport of neutral red can be dissociated into a "direct" mechanism, which is independent of droplet formation, and an "indirect" mechanism, in which concentration of the dye in droplet form within the cell is an essential feature (15). Under various experimental conditions, the dye can be observed to pass through the cells from the capillary to the tubule lumen without droplet formation; or on the other hand the dye can be observed to pass from

[3] The terms "secretion" and "absorption" are used here purely in a descriptive sense to indicate the direction of the processes by which the renal tubule passes material through its wall. Other definitions are entirely possible and the reader will find a full discussion of this semantic problem in *Tr. Conf. Renal Function*, October 20–21, 1949, Josiah Macy, Jr. Foundation, New York, 1950.

droplets to the lumen without concurrent entrance of it into the cells from the capillaries. Passage of dye by the "direct" mechanism may be increased or decreased by the action of electrolytes and of poisons which have no effect on droplet formation. In the case of neutral red, it appears that the "direct" mechanism is of greater quantitative importance than the "indirect" mechanism in the total process of secretion.

It is reasonable to suppose that a similar, "direct" mechanism may be of considerable importance in the absorption of hemoglobin, and in this light we may view the diffuse staining by benzidine of the cellular protoplasm found in control animals as an indication of hemoglobin passing through the cell in non-droplet form.[4] To speculate further, the effect of renin might decrease the absorption of hemoglobin by this "direct" process, without significant effect upon the "indirect" process of droplet formation. This would be analogous to the demonstrated effect of bichromate upon the secretion of neutral red.

Further light upon the mechanism of hemoglobin absorption might well be shed by an investigation of this problem in the isolated, perfused frog's kidney by the extravital method, a procedure which allows a correlation of the structural and functional aspects in more detail. The present experiments show clearly that the absorption of a protein, hemoglobin, may be affected in at least two manners: (*a*) by mechanisms having to do with the formation of droplets in the cells and (*b*) by mechanisms involving no alteration of the intracellular droplets.

SUMMARY

1. Administration of renin to the rat causes an increase in the glomerular permeability to hemoglobin and a decrease in tubular absorption of this substance as measured by functional methods.

2. Morphologic examination in the rat and in the rabbit reveals an increased concentration of hemoglobin in the tubular lumina, confirmatory evidence of increased permeability. However, there is no significant effect upon the formation of hemoglobin droplets within the renal cells.

3. By analogy with the events observed in neutral red "secretion," it is suggested that hemoglobin is absorbed by both a "direct" and an "indirect" mechanism and that renin administration affects or depresses the "direct" mechanism only.

BIBLIOGRAPHY

1. Pickering, G. W., and Prinzmetal, M., *J. Physiol.*, 1940, **98**, 314.
2. Brandt, J. L., and Gruhn, J. G., *Am. J. Physiol.*, 1948, **153**, 458.

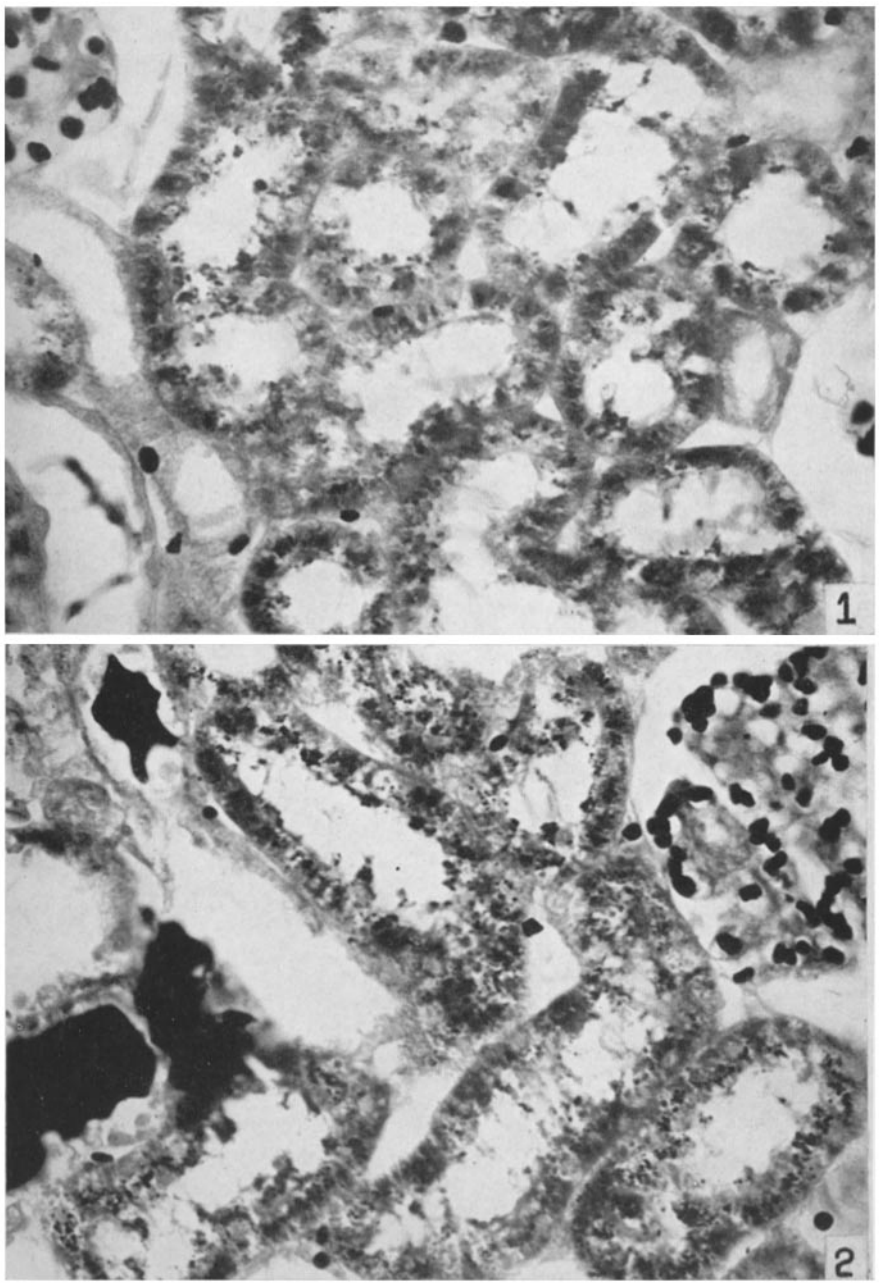
[4] It is becoming more generally assumed (16–21) that a small amount of protein constantly filters through the glomerular membrane and is absorbed by the tubules, so that the bladder urine is normally nearly free of protein. If this is so, the absorption of protein must be in non-droplet form, as droplets are not present in the "normal kidney."

3. Addis, T., Barrett, E., Boyd, R. I., and Ureen, H. J., *J. Exp. Med.*, 1949, **89,** 131.
4. Rather, L. J., and Addis, T., *J. Exp. Med.*, 1950, **91,** 567.
5. Lippman, R. W., Ureen, H. J., and Oliver, J., *J. Exp. Med.*, 1951, **93,** 325.
6. Friedman, M., *Am. J. Physiol.*, 1947, **146,** 387.
7. Lippman, R. W., *Am. J. Physiol.*, 1948, **152,** 27.
8. Addis, T., and Gray, H., *Growth*, 1950, **14,** 49.
9. Evelyn, K. A., and Malloy, H. T., *J. Biol. Chem.*, 1938, **126,** 655.
10. Bonsnes, R. W., and Taussky, H. H., *J. Biol. Chem.*, 1945, **158,** 581.
11. Fisher, R. A., Statistical Methods for Research Workers, New York, Hafner Publishing Co., Inc., 1950.
12. Oliver, J., *J. Mt. Sinai Hosp.*, 1948, **15,** 175.
13. Ralph, P. H., *Stain Techn.*, 1941, **16,** 105.
14. Rather, L. J., *J. Exp. Med.*, 1948, **87,** 163.
15. Oliver, J. R., and Lund, E. M., *J. Exp. Med.*, 1933, **57,** 435.
16. Addis, T., Glomerular Nephritis, New York, The Macmillan Co., 1948.
17. Dock, W., *New England J. Med.*, 1942, **227,** 633.
18. Rather, L. J., *Stanford Med. Bull.*, 1948, **6,** 117.
19. Gilson, S. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72,** 608.
20. Oliver, J., *Am. J. Med.*, 1950, **9,** 88.
21. Lippman, R. W., Urine and the Urinary Sediment, Springfield, Illinois, Charles C. Thomas, in press.

EXPLANATION OF PLATE 52

FIG. 1. Section of renal cortex of a control animal which had received an intravenous injection of hemoglobin and no renin. The cells of the renal epithelium of the proximal convolution are filled with small droplets of hemoglobin which, stained a bright yellow by the Ralph method, appear black in photographic reproduction. × 525.

FIG. 2. A similar section from an animal which had received an injection of hemoglobin with the addition of renin. The cells of the proximal convolution are equally well filled with small black-appearing droplets of hemoglobin. To the right, collections of concentrated hemoglobin are seen in the tubule lumina. × 525.



(Lippman, Ureen, and Oliver: Mechanism of proteinuria)